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(54) Title: THE PROCESS FOR THE EXPRESSION AND PRODUCTION OF THE RECOMBINANT HUMAN BETA-CIS INTERFERON

(57) Abstract

The present invention describes the new human protein interferon beta-cis isolated from human amniotic cells, its corresponding encoding recombinant DNA molecule and the process of production of the human recombinant protein interferon beta-cis produced through techniques of genetic engineering, to be used in human or veterinarian clinical or in research. This modified beta-cis recombinant interferon contains a substitution of the amino acid 60 from a tyrosine to a cysteine. The antiviral activity is maintained stable when compared with unmodified beta interferon.

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THE PROCESS FOR THE EXPRESSION AND PRODUCTION OF THE RECOMBINANT HUMAN BETA-CIS INTERFERON.

TECHNICAL FIELD OF THE INVENTION

The present invention refers to the general field of the technology of the DNA recombinant proteins, for the production of the protein of interferon beta - cis human mutant, to be used in human, veterinarian clinical or in research.

BACKGROUND OF THE INVENTION

Interferons (IFN) are a family of proteins whose first described biological activity was the ability to inhibit viral replication. Besides their antiviral action, IFNs possess a variety of other biological activities, controlling cell growth, differentiation, and modulating the immune system (Vilcek, J. & Sem, G.C.In *Virology*(Ed.) Fields, B.N., Knipe, D.M., Howley, P.M. 375-399, PA: Lippincott-Raven Publishers, 1996).

Humans IFNs are classified in two major sub-families based on their biological and physical properties. The type I IFN family includes fibroblast β-interferon (IFN-β), leukocyte α-interferon (IFN-α) with 15 or more genes and pseudo-genes and IFN-ω and IFN-τ. The type II IFN family is represented by the IFN-γ and it is produced by T lymphocytes and NK cells in response to mitogens and antigenic stimuli (Vilcek, J. & Sem, G.C.In ***Virology*** (Ed.) Fields, B.N., Knipe, D.M., Howley, P.M. 375-399, PA: Lippincott-Raven Publishers, 1996).

Like in humans, most of the mammalian species have large IFN-α gene families. The existence of multiple IFN-β gene families was shown in bovine

(three genes) whereas, humans and most of other mammalian species probably contain only one gene (Ryan, A M., Gallagher, D.M. $ Womack, J.E **Mammallian Genome**, 3(19): 575-578, 1992).

Placental tissues can produce IFNs. IFN activity has been detected in amniotic fluid, umbilical cord and blood of human placentas during pregnancy without clinical signs of viral (Lebon, P., Girard, S., Thepot, F.& Chany, C. **Journal of General Virology**, 59: 393-396, 1982). also in mouse placentary tissues (Fowler, A.K., Reed, C.D. & Giron, D.J. Nature, 285: 266-267, 1980). Ovine and bovine trophoblast cells produce another type of IFN named IFN-τ, during the embryo implantation into the uterus (Imakawa, K., Anthony, R.V., Kazemi, M., Mariotti, H., Poltes, H.G. & Roberts, R.M. **Nature**, 330: 377-379, 1987.). This IFN is necessary for fetal implantation, acting as an anti-luteolysin, resulting in the continued secretion of progesterone (Cross, J.C. & Roberts, R.M. *Proceedings of the National* **Academy of Sciences of the USA**, 88: 3817-3820, 1991). Recently a human trophoblast IFN (hTIFN) cDNA, was described by (Whaley, A.E., Meka, C.S.R., Harbison, L.A., Hunt, J.S.& Imakawa, K **The Journal of Biological Chemistry**, 269:10864-10868, 1994) whose nucleotide sequence is similar (85%) to ovine and bovine IFN-τ. The HuIFN-τ and HuIFN-ω mRNAs were detected in the cytotrophoblast cells during all the pregnancy. This fact suggests that these IFNs are important as a first line of defense, protecting the fetus from virus transmission and also participating in the progressive changes of placental development (Whaley, A.E., Meka, C.S.R., Harbison, L.A., Hunt, J.S.& Imakawa, K **The Journal of Biological Chemistry**,

269:10864-10868, 1994). Human trophoblast cultures produced IFN-β, IFN-αI and tau interferon when infected by virus (Aboagye-Mathiesen, G., Thóth, F.D., Juhl, C.H., Norskov-Lauritsen, N.N., Petersen, P.M. & Ebbesen, P. **Journal of General Virology**, 71: 3061-3066, 1990 and Thóth, F.D., Norskov-Lauritsen, N.N., Juhl, C.H, Aboagye-Mathiesen, G. & Ebbesen, P. **Journal of General Virology**, 71: 3067-3069, 1990).

IFN are ligated to receptors located at the cellular surface, activating in this way the transduction of citoplasmic signals that will induce in the nucleus a series of responsible genes for the activities of IFN (JOKLIK, W.K. ,Interferons, In: FIELDS, B.N.AND KNIPE,D.M. (Ed.)**Virology**, 2a. Ed. -New York, Raven Press Ltd., cap.16, p.383-410, 1990).

All the members of the family of IFNs α possess a sequence of 165 to 166 a.a., of the which 4 cysteins (Cys) are highly conserved in the positions 1; 29; 98; 99 or 100; 138 or 139; and they are responsible for the dissulfete bridges (S-S). The ligation S-S between Cys 1; 98; 99 or 100 and between Cys 29 and 138 or 139 are important in the stabilization of the structure of the IFN (WETZEL, R. et al., **J. Interferon Rês.**, 1:381-389,1981). The ligation among these two Cys is indispensable in the maintenance of the biological activity of this IFN (PATH, T: ,EMBO **J.**, 11: 3193-3201 et al., 1992).

IFN β is produced by fibroblasts and it is a glicoprotein with relative molecular mass of 22-23 kDa and specific antiviral activity of $2\text{-}5x10^8$ international units (protein UI)/mg. This IFN is coded by an gene located in the chromosome 9, it doesn't possess introns and it codes a protein of 166 a.a., IFN β of bovine, suínos and caprinos are coded by multiple genes (OF MAEYER, E.M. and OF MAEYER-GUIGNARD, J. - **Interferon and other regulatory cytokines**. John Wiley and Sounds, New York, 1988).

IFN β possesses 3 cystein residues (Cys) in the positions 17, 31 and 141. Two Cys 31 and 141 form a dissulfite bridge while Cys 17 stays free. The studies (MARK, D.F., et al., **Proc. Natl. Acad. Sci. It USES,** 81: 5662-5666.

1984), in which the Cys 17 were changed for a serine showed that this IFN presented the same spectrum of biological activities of IFN β such as the anticelular activity, antiproliferativ, activation of cells NK and neutralization .ac anti HulFN and presents a larger stability than HulFN naturalβ when incubated for -70°C.

In other experiment, SHEPARD,H.M. et al., **Nature**, 294:563-565, 1981, changed the Cys 141 for a tyrosine , and renders an biologically inactive molecule.

The mechanisms through which IFNs induces the state antiviral have been studied enough. It is accepted that the activation of the enzyme 2'5 ' oligoadenilato sintetase and of the RNA dependent protein kinase (dsRNA) are important in the antiviral action (PESTKA, S., et al., **Ann. Rev. Biochem.**, 56:727-777, 1987).

It is an object of the present invention to describe the new human protein interferon beta-cis isolated from human amniotic cells, their corresponding encoding recombinant DNA molecule and the process of production of the human recombinant protein interferon beta-cis produced through techniques of genetic engineering, to be used in human or veterinarian clinical or in research.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1:The vector used for the expression of the interferon beta cis.
Figure 2:Nucleotide sequence of the interferon beta cis
Figure 3:Expression of beta cis interferon in *E. coli*
Figure4:Purification of beta cis interferon
Figure 5:Induction of genes

DETAILED DESCRIPTION OF THE INVENTION

In order that this invention may be better understood the follow examples for illustrative purposes only, are described. The examples ilustrate the present invention and are not intended to limit it in spirit or scope. The process can be understood better through the following description in consonance with the examples

EXAMPLE 1

Primary culture of the human amniotic cells (1)

Primary culture of the human amniotic cells was established by using amniotic membranes that were separated from the placenta , washed in a saline solution and cut in fragments of approximately 1 cm^2.The fragments were digested with tripsin (0,25 -0,30%) in the proportion of 5 to 7 ml per gram of tissue. After each stage of treatment with trypsin the cells were centrifuged and cultured in minimum essential medium containing salts of Eagle at the density of 15 to 25x 10^6 cells per Petri dishes.

EXAMPLE 2

Induction of interferon (2)

One week after implantation the cells were infected with Sendai virus or Newcastle Disease Virus in the proportion of 3,0 to 4,8 x 10^{-6} hemaglutinant units of the virus per cell for 1 hour and again incubated during 4 at 8 hours.

.EXAMPLE 3

RNA extraction and purification (3,4,5,6,7.8)

For the RNA extraction the infected cells were washed with saline. and the disrupted with 3,5 to 4,5 ml of a 3 to 4 M guanidine isothiocyanat solution on ice bath during 10 to 20 min..For the purification of the RNA 1,5 to 2,0 ml of a solution of cesium chloride 5.7 to 5,8 M were added, and centrifuged for 114.000g during 18 at 24 hours to 20° C - 24° C. RNA was homogenized in a Tris - EDTA solution and the extraction was made with the same volume of phenol/cloroformio/ isoamilic alcohol in the proportion 25:24:1, shaken in vortex and centrifuged. RNA was precipitated with 1/10 of the volume of sodium

acetate 2 to 3 M pH 5.5 to 6,0 and ethanol, centrifuged and the pellet ressuspended in water.

EXAMPLE 4

cDNA synthesis (9,10)

5 to 10 ug of RNA was used as template for the synthesis of the first cDNA strand using, 0,5 to 1 ug of primer T15, pyrophosphato 40-50 mM, 20 to 30 U of avian reverse transcriptase and its buffer.

The amplification of the fragment corresponding to the interferon starting from the cDNA obtained in the stages 1, 2, 3, 4 ,5 6,7,8,9 or starting from the vector that contains the cloned DNA of the interferon gene beta-cis was carried out using specific oligonucleotides (5'GCCGGATCCTACAACTTGCTT GGATTCCTA3 and 5'GCCAAGCTTAGTTTCGGTCATTCCTGTAAGTC3 ')for the region of the corresponding fragment of the protein interferon beta that contains the for restriction enzymes BamH 1 and Hind III, in the reaction in chain of the polimerase (PCR).

The reaction was made with Taq polimerase buffer (500 mM KCl, 100 mM Tris-HCl pH 9,0-9,5, 1,5-2,5 mM $MgCl_2$ and 1-2% triton X-100), 0,1-1 U of Taq polimerase (Promega ,E.U.A., Cat. no. M186A), 0,5-1,5 mM $MgCl_2$, 20-50 mM of each nucleotide (dATP,dCTP,dGTP,dTTP) 10-30 pmoles of each primer, and 0,01a 0,1 ng cDNA and H_2O distilled and sterile q.s.p. 50-100 µl. The reaction was displayed in 1-2 cycles at 94-96°C/1-2 min; 53 to 55°C/1 -2 min.; 70-72°C /1 - 2 min; 30 cycles at 94-96°C/1 to 2 min; 36 -38°C/1-2min; 70-72°C/1-2 min and more 1 cycle to 94-96°C/1-2 min; 36 -38°C/1 to 2 min; 70-72°C/10-15 min.

EXAMPLE 5

Fractionation and purification of DNA(11,12)

The fractionation of the DNA was accomplished in eletrophorese in agarose gel 1,5-2,0% .The purification of the amplified DNA was made by cutting out the band of the gel. The band was diluted in 2-3 times the volume of NaI solution (NaI 8M + 0,022 M DTT) and sodium phosphate buffer (1M pH 6.0-

6,5) and incubated for 5-10 min. to 50-56°C. Glass spheres were added to the suspension, mixed , incubated 1-5 min. at room temperature and centrifuged 10-30 seconds . The spheres were washed with Etanol buffer(75% of Etanol, 0.01 M Tris-HCl, pH 7,0-7,6, 0,01 M EDTA, pH 8,0-8,5). The DNA was eluted from the glass spheres i with buffer(Tris pH 7,0-7,4 10 mM, 1-3 mM EDTA) to 50-56° C for 1-5 min.

EXAMPLE 6

Digestion of DNA fragment and cloning(13,14)

For the digestion of the DNA this was firstly treated with enzyme Hind III in a reaction with 10-20 U of Hind III (Biolabs, England), 3-5 µl bufferI (Promega.EUA) and H_2O distilled qsp 30-50 l with incubation for 37oC (I Take a bath Maria) for 2-4 h. Soon after it was added to the tube of the reaction 10-20 U of Bam HI (Biolabs, England), 5-10 µl of react III (BRL, USA), H_2O_{dd} qsp 50-100 µl and incubation at 37°C for 2-4 h. For cloning of the DNA fragment in the plasmid PDS-56, the digestion of the vector was accomplished with the restriction enzymes Hind III and Bam HI in a reaction contents vector, 10-20 U of enzyme Hind III(Promega, USA), 2-5 (lid I B (Promega,E.U.A.), H_2O distilled qsp 20-50µl. with incubation at 37°C for 2-4 h. Later 10-20 U of the enzyme Bam HI (Promega, USA), 5-10 µl of react III (BRL, E.U.A), H2O distilled qsp 50-100µl were added to the reaction and incubated at 37°C for 2-4 h. The product of this digestion was analized in eletrophorese in a 1% agarose-TAE gel .The band corresponding to the digested plasmid was cutted out of the gel and transferred to a eppendorf tube (1,5 ml) and purified.

EXAMPLE 7

Ligation , transformation and clone selection(15,16,17)

In the ligation reaction of the DNA fragment 20-50 ngof the insert was added to 5-15 ng of the vector, 0,5-2,0 U of T4 ligase (Promega, USA), ATP 5 mM (Promega,E.U.A.), ligation buffer(Promega,E.U.A.), H_2O dd qsp 15µl, with

incubation at 14-16°C (BOD, FANEN, Brazil) for 12-18 h.

The bacterial transformation was done with *Escherichia coli* bacteria. The volume of the ligation reaction was completed to 40-60µl with buffer(Tris 10 mM pH 7,2-7,4, EDTA 1 mM) and added of 100 µl of suspension of competent bacteria. The tubes were slightly agitated and immediately incubated in bathing of ice bath 20-40 min , submitted to a thermal shock at 40-42°C for 1-3 min. and again in ice bath for 20-40 seconds.Medium LB (Bacto triptona 1% p/v, extract of yeast 0,5% p/v, NaCl 171 mM) without antibioticswas added at the double of the volume and incubated for 37°C by 1-2 h. The bacteria were sedimented, homogenized in LB and inoculated in Petri plates with LB ágar (ágar 1,5% p/v, yeast extract 0,5% p/v, triptone 0,1% p/v, NaCl 0,5% p/v pH 7,2-7,5) with 50-200 µg/ml ampicilin and 20-100 µg/ml Kanamicine. The plates were incubated at 37°C for 15-24 h. For the selection of the positive clones they were grown in LB with 50-200 µg/ml ampicilin and 20-100 µg/ml Kanamicine at 37°C under agitation for 15-20 h. After incubation a reaction in chain of the polimerase using specific primers of the vector (for amplification of the area corresponding to insert) being the primer (sense) 5'TTCATTAAAGAGGAGAAATT3' and primer(anti-sense) 5'CTATCAACAGGAGTCCAAGC3'. The reaction was made with Taq. polimerase buffer10 X (KCl 500 mM, Tris-HCl 100 mM pH 9,0-9,5, $MgCl_2$ 15-25 mM and triton X-100 1-2%), 0,5-1,0 U of Taq polimerase (Promega, USA) ,0,5-1,5 mM $MgCl_2$, 20-50 mM of each nucleotid (dATP, dCTP, dGTP, dTTP), 10-30 pmoles of each primer, 0,5-1µl of bacterial suspension and H_2Odd sterile qsp 20-40µl. The reaction was processed with 1-3 cycles for 94-96°C/5min., 50-55°C/1-2 min., 70-72°C/1-2 min.; 30 cycles to 94-96°C/30-45 seg., 45-50°C/30-45 seg., 70-72°C/30-45 seg.e 1 cycle to 94-96°C/1-2 min., 45-50°C/1-2 min., 70-72°C/10-15min. The product of this reaction was fractioned in eletrophorese in agarose gel at 1-2%.

EXAMPLE 8

Sequencing (18)

The positive clones were sequenced to found the mutant interferon beta-cis.

EXAMPLE 9

Protein production , induction and lysis of bacteria(19,20,21)

The positive clones for the mutant interferon beta-cis were used for the production of the protein and they were grown in LB medium with 50-200 µg/ml ampiciline and 50-200 of Kanamicine µg/ml and incubated at 37°C under agitation until the optical density (OD 600 nm) of 0,5-0,7 when for the induction of the protein (20) IPTG(Isopropyl-(-D-thiogalacoside) to 0,2-0,4 M was added and incubated for 3-5 h. The bacterias (21) were centrifugated and the supernatant was discarded and the sediment homogenized in buffer A(Guanidine-HCl 5-6 M, sodium phosphate 0,1-0,2 M, Tris 0,01-0,02 M pH 7,8-8.0) with agitation for 1-2 h.

EXAMPLE 10

Protein purification (22)

After it was centrifuged the supernatant was applied to a column with resin of Ni-NTA. For the purification of the protein the column was washed sequencially with buffer A, buffer B (Urea 7-8 M, phosphate of sodium 0,1-0,2 M, Tris 0,01-0.02 M pH 7,8-8,0) .e with buffer C (Urea 7-8 M, phosphate of sodium 0,1-0,2 M. Tris 0.01-0.02 M pH 7,0-7,2). The protein was eluted with buffer D (Urea 7-8 M. sodium phosphate 0.1-0.2 M. Tris 0.01-0,02 M pH 5,0-5,2) and sequencially with (Urea 7-8 M, phosphate of sodium 0,1-0,2 M, Tris 0,01-0,02 M pH 4.0-4.2); fractions and a sample of 50µl were collected andeach fraction was diluted v/v in sample buffer, heated for 10 min. and submitted to eletrophoresis in polyacrilamida gel (SDS-PAGE). The gel was analyzed for the presence of the fraction that just contained the corresponding band to the purified protein interferon beta-cis.

While the present invention has been described in connection with examples. it will be understood that modifications and variations apparent to

those ordinary skill in the art are within the scope of the present invention.

1. WHAT IS CLAIMED IS:

Process for production of the human recombinant protein interferon beta-cis comprising the following steps:

(1) primary culture of human amnioticcells

(2)infection with the virus

(3)Obtention of RNA

(4) Lysis of the cells

(5) Fractionation of RNA

(6) Precipitation of RNA

(7) extraction of RNA

(8) precipitation of RNA

(9) synthesis of first strand cDNA

(10) amplification of the DNA

(11) Fractionation of the DNA

(12) purification of the DNA

(13) digestion of the DNA

(14) Cloning

(15) ligation of the DNA to the vector

(16) transformation of the competent bacterias

(17) selection of the positive clones

(18)Sequencing of the clones

(19) production of the protein

(20) induction of the bacteria

(21) Lysis of the bacteria

(22) purification of the protein

2.The procees according to claim 1 the fact that the stages: (1), (2), (3), (4), (5), (6), (7), (8), (9), (10), (11), (12), (13), (14),(15), (16),(17),(18), (20),(21) they can be optional.

3 Process for production of the human recombinant protein interferon beta-cis in agreement with the claim 1, characterized by the fact that the primary cultivation

of cells amnióticas (1) it is obtained separation of the cells of the membrane amniótica through by tripsina action or other agent proteolitico.

4.Process for production of the human recombinant protein interferon beta-cis de agreement with the claim1, characterized by the fact that the infection of cells is done with the virus Sendai or other virus ,ou drugs that stimulates the interferon production in the cell

5.Process for production of the human recombinant protein interferon beta-cis in agreement with the claim 1, characterized by the fact that the mRNA obtaining (3) it is done through the wash of the monocamada of cells with saline or any buffer with pH and physiologic saline concentration and the lysis of the cells (4) it is done with guanidine isotyocyanate or other physical-chemical process with lithic properties

6.Process for production of the human recombinant protein interferon beta-cis in agreement with the claim 1, characterized by the fact that the transformation of the competent bacterias (16) it is done with bacterias *Escherichia coli* or any other microorganism

7.Process for production of the human recombinant protein interferon beta-cis,de agreement with the revindication 1, characterized by the fact that the purification of the protein (22) it is done in a column of nickel chelate or any any other method that allows the purification of the protein.

8. Human recombinant protein interferon beta-cis manufactured through the demanded process of 1-23, characterized by the fact of presenting in its structure the following aminoácidos: MSYNLLGFLQ RSSNFQCQKL LWQLNGRLEY CLKDRMNFDI PEEIKQLQQFQKEDAALTIC EMLQNIFAIF RQDSSSTGWN ETIVENLLAN VYHQINHLKTVLEEKLEKED FTRGKLMSSL HLKRYYGRIL HYLKAKEYSH CAWTIVRVEILRNFYFINRL TGYLRN

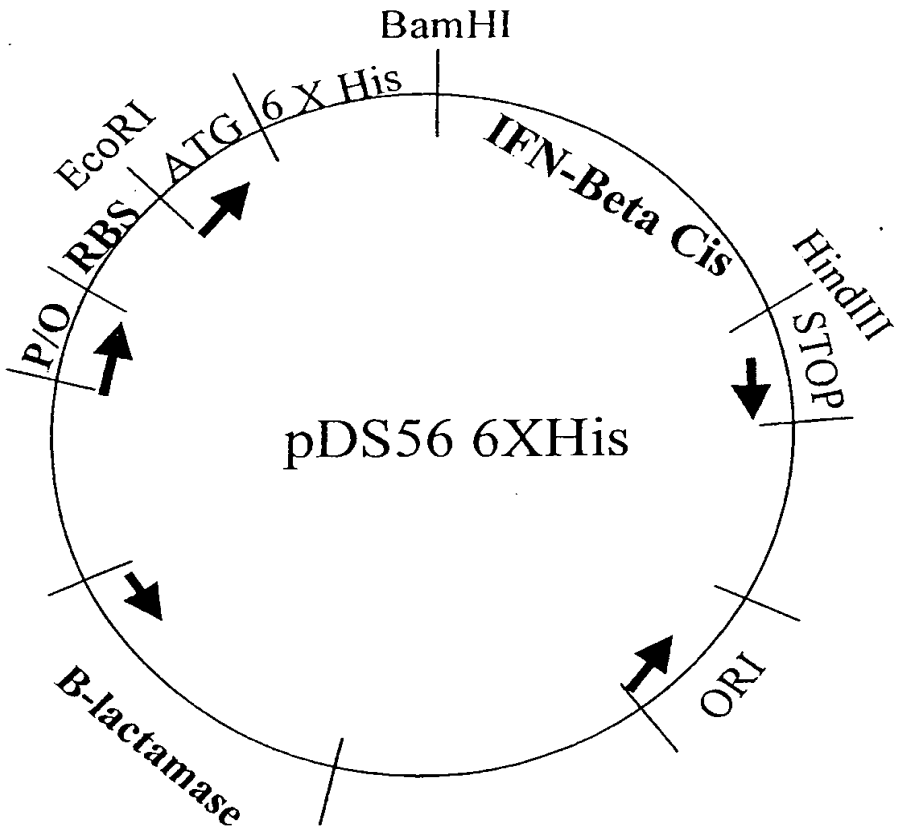
BamHI
6 X His
ATG
EcoRI
RBS
P/O
IFN-Beta Cis
HindIII
STOP
pDS56 6XHis
ORI
B-lactamase

Figure 1

```
ATGAGCTACAACTTGCTTGGATTCCTACAAAGAAGCAGCAATTTTCAGTGTCAG
MetSerTyrAsnLeuLeuGlyPheLeuGlnArgSerSerAsnPheGlnCisGln

AAGCTCCTGTGGCAATTGAATGGGAGGCTTGAATACTGCCTCAAGGACAGGATG
LysLeuLeuTrpGlnLeuAsnGlyArgLeuGluTyrCysLeuLysAspArgMet

AACTTTGACATCCCTGAGGAGATTAAGCAGCTGCAGCAGTTCCAGAAGGAGGAC
AsnPheAspIleProGluGluIleLysGlnLeuGlnGlnPheGlnLysGluAsp

GCCGCATTGACCATCTGTGAGATGCTCCAGAACATCTTTGCTATTTTCAGACAA
AlaAlaLeuThrIleCysGluMetLeuGlnAsnIlePheAlaIlePheArgGln

GATTCATCTAGCACTGGCTGGAATGAGACTATTGTTGAGAACCTCCTGGCTAAT
AspSerSerSerThrGlyTrpAsnGluThrIleValGluAsnLeuLeuAlaAsn

GTCTATCATCAGATAAACCATCTGAAGACAGTCCTGGAAGAAAAACTGGAGAAA
ValTyrHisGlnIleAsnHisLeuLysThrValLeuGluGluLysLeuGluLys

GAAGATTTCACCAGGGGAAAACTCATGAGCAGTCTGCACCTGAAAAGATATTAT
GluAspPheThrArgGlyLysLeuMetSerSerLeuHisLeuLysArgTyrTyr

GGGAGGATTCTGCATTACCTGAAGGCCAAGGAGTACAGTCACTGTGCCTGGACC
GlyArgIleLeuHisTyrLeuLysAlaLysGluTyrSerHisCysAlaTrpThr

ATAGTCAGAGTGGAAATCCTAAGGAACTTTTACTTCATTAACAGACTTACAGGT
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TACCTCCGAAACTGA
TyrLeuArgAsnEnd
```

Figure 2

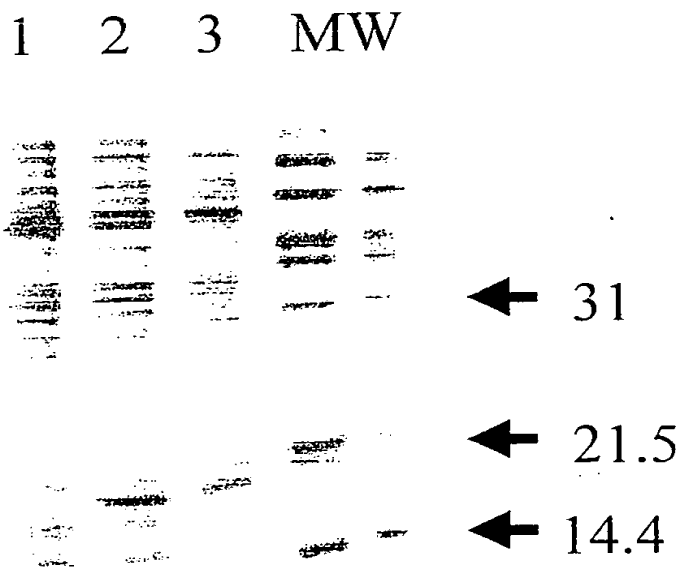

Figure 3

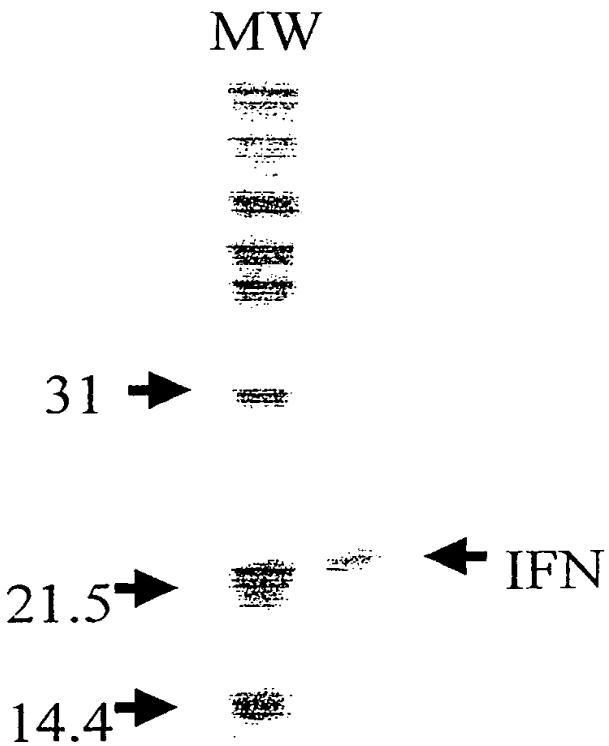

Figure 4

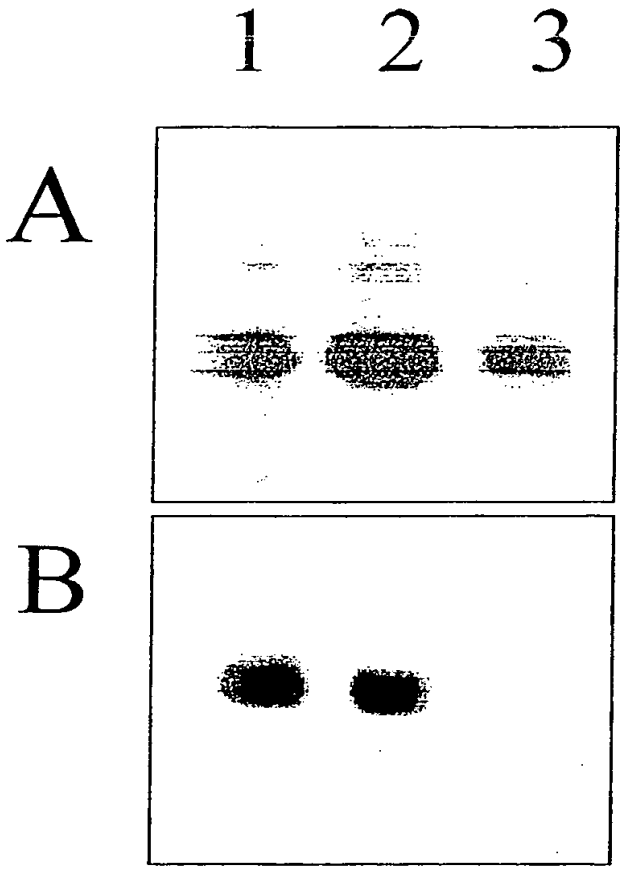

Figure 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/BR 97/00082

A. CLASSIFICATION OF SUBJECT MATTER

IPC^6: C 12 N 15/22

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC^6: C 12 N 15/22

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 571 743 A1 (TANIGUCHI) 01 December 1993 (01.12.93), abstract; page 2.	1
A	EP 0 355 202 A1 (TANIGUCHI) 28 February 1990 (28.02.90), page 2; claims 1-3.	1

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 April 1998 (16.04.98)	29 April 1998 (29.04.98)
Name and mailing address of the ISA/ AT AUSTRIAN PATENT OFFICE Kohlmarkt 8-10 A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer Wolf Telephone No. 1/53424/436

Form PCT/ISA/210 (second sheet) (July 1992)

CID: <WO___9827211A1_I_>

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/BR 97/00082

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